Eur. J. Clin. Chem. Clin. Biochem.
Vol. 31, 1993, pp. 749–752

Application of the Time-Resolved Immunofluorometric Assay to the Study of C3 Complement Component Glycation *in vitro* and *in vivo*

By *O. Gaillard*[1], *Dominique Meillet*, *Michèle Bordas-Fonfrède*[2], *Lina Khalil*[2], *Jeanne Galli*[1] and *J. Delattre*[2]

[1] *Laboratoire d'Immunochimie*
[2] *Laboratoire de Biochimie*
Hôpital de la Salpêtrière, Paris Cédex 13, France

(Received May 10/August 6, 1993)

Summary: A time-resolved immunofluorometric assay (TR-IFMA) was used for the measurement of glycated C3. The very high sensitivity of this technique allowed the direct measurement of glycated and non-glycated proteins (especially C3) in chromatography eluates. C3 glycation in vitro after incubation with 20 mmol/l glucose was always less than 3.5% by day 5. As determined with the TR-IFMA, the means ± standard deviations of glycated C3 were 0.20% ± 0.04 for non-diabetic subjects and 0.88% ± 0.06 for insulin-dependent diabetic patients. The low percentages of glycated C3 in both our in vitro and in vivo studies show that this protein is subject to only moderate rates of glycation.

Introduction

There is now evidence that plasmatic proteins show high percentages of glycation in diabetic patients (1, 2). Various contradictory studies concerning the consequences of this glycation on the functional properties of proteins especially those involved in immune mechanisms have been published (3–6).

The C3 complement component, the "main element" of the complementary system, is involved in many immune mechanisms, especially the transport and the elimination of circulating immune complexes (7–9).

Austin et al. (10) reported relatively high levels of C3 glycation in both healthy subjects and diabetic patients. These results are surprising, given the short half-life of the protein (48 hours).

It is of interest to investigate the possible effect of C3 glycation on immune mechanisms involving this component. Therefore, the objective of the present study was to evaluate the time course of C3 complement glycation in vitro and the percentages of glycated C3 in both healthy subjects and diabetic patients.

Prior to their assay, glycated and non-glycated fractions of C3 complement must be separated by boronate affinity chromatography. Because of the low concentration of C3 in the eluates, *Austin* et al. (10) performed a preliminary concentration by lyophilization, before measurement by immunoturbidimetry. To avoid this concentration step, a very sensitive time-resolved immunofluorometric assay (TR-IFMA) was proposed. Its very high sensitivity was reported in a previous study (11), and it was used in the present study for the direct measurement of C3 in chromatography eluates.

Materials and Methods

Study population

Two groups of patients were studied:

The first group consisted of 30 non-diabetic healthy subjects (15 men; 15 women; average age: 37 years, range 18–60), whose glycaemia ranged from 4.5 to 6.5 mmol/l, and with no impairment of post-absorption glycaemia.

The second group consisted of 30 diabetic insulin-dependent patients (15 men; 15 women; average age: 48 years; range: 20–74) whose glycated haemoglobin ranged from 6.8 to 13.7% (mean = 9.9, SD = 1.6). Blood samples were collected by venipuncture in EDTA-containing tubes, and in evacuated blood-collection tubes without anticoagulant. Plasma and sera were immediatly centrifuged at 500 *g* and stored at −20 °C until the assay.

Separation of glycated and non-glycated proteins

Proteins were separated into glycated and non-glycated fractions by boronater affinity chromatography (Glyco-Gel™ Analytical Columns-Pierce, Rockford, IL) as previously described (12–14), equilibrated with ammonium acetate 0.25 mol/l, magnesium chloride 0.005 mol/l buffer, pH 8. Non-glycated proteins were eluted with 20.2 ml of the same buffer. Glycated proteins were eluted with 3 ml of sodium citrate buffer 0.2 mol/l, pH 4.5.

Protocol of in vitro glycation

Pooled sera from non-diabetic patients (total C3 concentration = 0.67 g/l) were immediately divided in 3 aliquots of 5 ml each (A, B, C). Fraction A was incubated for up to five days at 37 °C in the presence of sodium-potassium phosphate buffer 0.05 mol/l, pH 7.5, NaN_3 300 mg/l. Fractions B and C were incubated with 66 mmol/l sodium-potassium phosphate buffer, pH 7.5, containing 1 g/l NaN_3 as preservative, and glucose (20 mmol/l for A, 500 mmol/l for B). Two ml of each fraction were taken at day 0, 2, 3 and 5, and immediately dialysed against NaCl 0.15 mol/l for 24 hours at 4 °C. Proteins were then separated into glycated and non-glycated fractions as described previously. Concentrations of glycated and non-glycated C3, albumin and immunoglobulins (IgG) were determined.

Measurement of total C3 and glycated and non-glycated C3 fractions with TR-IFMA

Concentrations of C3 in glycated and non-glycated fractions were measured using TR-IFMA. This method was described previously (11). Briefly, it includes the europium-labelling (Kabi-Pharmacia, Uppsala, Sweden) of an antibody to human C3c (Dako, Copenhagen, Denmark) and the immunoassay procedure. Polystyrene microtitre plates (Microwell™, Nunc Inc., Naperville, IL) were coated by adding 200 µl of unlabelled antibody to C3c solution. The wells were then washed three times with 250 µl of a saturation solution (50 mmol/l $NaH_2PO_4 \times 2\,H_2O$ containing 60 g of sorbitol and 0.5 g of bovine serum albumin per litre) per well to block non-specific binding sites. After incubation of sera diluted to 10^{-3} and 10^{-2}, glycated eluates diluted to 10^{-2} and 10^{-1} and non-glycated eluates diluted to 10^{-3} and 10^{-2}, then washing, the wells were incubated with the conjugate. The amount of labelled antibody bound to C3 was quantified by first dissociating europium from the antibody with enhancement solution. The fluorescence of the formed europium 2-naphthoyl-tri-fluoro-acetone acetate was measured as counts per second in a time-resolved fluorometer (Arcus™ 1230, Wallac Oy, Turku, Finland).

Other assays

HbA_{1c} was measured using high performance liquid chromatography (Diamat™, Biorad, Richmond, CA). Fructosamine was measured using the colorimetric absorption of reduced nitroblue tetrazolium (fructosamine test plus™, Roche, Bâle, Switzerland). Immune complexes were measured using the solid phase C1q binding immunonephelometric assay (BNA™, Behring, Marburg, Germany). The glycated and non-glycated albumin and IgG fractions were measured in the eluates using immunonephelometric assay.

Statistical analysis

Percentages of each specific glycated protein (C3 complement, albumin and IgG) were compared between the two groups using the *Mann & Whitney* non-parametric test. Correlations between parameters were studied with the *Spearman* rank correlation test.

Results

In vitro glycation

The time course of the percentage glycation of C3, albumin and IgG is presented in figure 1. Without prior incubation of pooled sera with glucose, the percentage of glycated C3 was very low, always lower than 1%, and showed very little variation. Incubation of pooled sera with 20 mmol/l glucose resulted in a very low percentage of glycated C3: 1.5 and 3.4% at day 2 and day 5, respectively. However, with a solution of 500 mmol/l glucose, the percentage of C3 glycation was relatively high (12.5%) at day 2 and reached 63.3% at day 5. It was noteworthy that the percentages of glycated albumin were close to those of glycated C3 after incubation with 20 mmol/l glu-

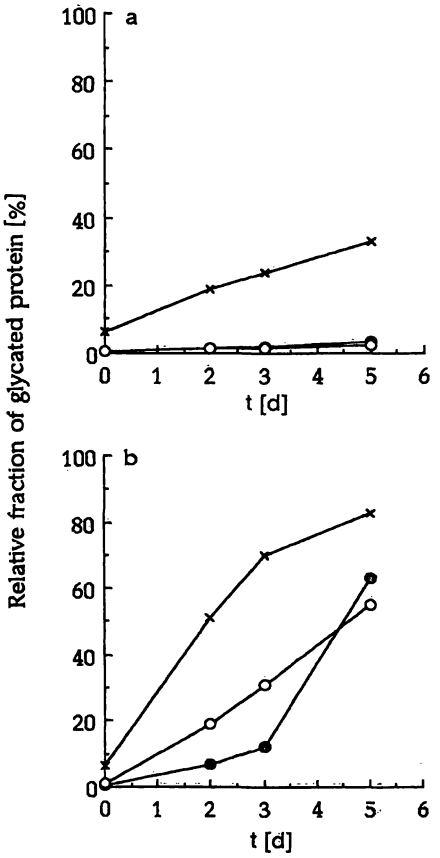

Fig. 1. Time course of glycation of C3 complement, albumin and IgG following incubation of serum in vitro for periods of 0 to 5 days in buffer containing
a) 20 mmol and
b) 500 mmol of glucose per litre at pH 7.5
—●— C3 complement, —o— albumin, —x— IgG

cose (1.3 at day 2 and 2.6 at day 5), in contrast to the percentages of glycated IgG which were already high after incubation with 20 mmol/l glucose (19% at day 2 and 33% at day 5). It was noteworthy that the percentage of glycated IgG was 83% at day 5 after incubation with 500 mmol/l glucose.

In vivo study

Table 1 gives the means and standard deviations for the percentages of glycated C3, other glycated quantities, and the concentrations of total C3, and immune complexes measured in each group.

Tab. 1. Comparison of means of immune complexes, C3 complement, glycated C3 and other glycated proteins between healthy subjects and diabetic patients

	Healthy subjects (n = 30) $\bar{x}^a) \pm SD^b)$	Diabetic patients (n = 30) $\bar{x}^a) \pm SD^b)$
Immune complexes (mg/l)	1.42 ± 0.71	1.63 ± 1.38
C3 complement (mg/l)	607 ± 127	640 ± 105
Glycated fractions (%)		
C3	0.20 ± 0.04	0.88* ± 0.6
IgG	6.01 ± 1.25	13.89* ± 5.27
Albumin	0.87 ± 0.15	3.37* ± 0.99
Fructosamine (μmol/l)	214 ± 25	364* ± 80
HbA_{1c} (%)[c])	4.85 ± 0.30	9.69* ± 1.85

[a]) Mean values
[b]) Standard deviation
Statistical comparison between the mean of studied quantities in healthy and patient groups (*$p < 0.001$); *Mann & Whitney*'s test
[c]) Fraction of haemoglobin

The percentages of glycated C3 were significantly higher in diabetic patients than in healthy subjects ($p < 0.001$) (0.88% and 0.20%, respectively). Nevertheless, there was no significant difference between groups for the concentrations of total C3 and immune complexes.

The percentages of glycated C3 and HbA_{1c} were significantly correlated ($p < 0.01$). A lower correlation was found between the percentages of glycated C3 and glycated albumin ($p < 0.05$). There was no significant relationship between the percentages of glycated C3 and those of glycated IgG and fructosamine respectively.

Discussion

In view of the low concentrations of glycated C3, a TR-IFMA was used in the present study. Its very high sensitivity was described in a previous study (11). This method allows the direct measurement of components in chromatography eluates and therefore avoids the preliminary step of concentration.

After incubation with 20 mmol/l glucose, the percentage of glycated C3 was very low at day 2 and was always less than 3.5% by day 5. The time course of C3 glycation was close to that of albumin glycation. The relatively high percentage of glycated IgG found in the present study was also reported by *Dolhofer-Bliesener* et al. (15). However, the high percentages of glycated C3 (higher than 60% at day 5) found with 500 mmol/l glucose were close to those found by *Austin* et al. (10). Moreover, the percentages of glycated albumin and IgG described in the present study are in agreement with those reported by *Austin* et al. (10).

The low percentage of glycated C3 observed in vitro with 20 mmol/l glucose was also found in in vivo experiments, both in non-diabetic subjects and diabetic patients (0.20% and 0.88%, respectively). However, in comparison with non-diabetic subjects, the percentage of glycated C3 increased significantly in diabetic patients. *Austin* et al. (10) also reported such a difference, although the percentages they found were very different to ours (about ten times higher).

Such divergences between the two studies could be perhaps explained by the prior step of lyophilization used by *Austin* et al. (10).

The low percentages of glycated C3 found in the present study appear more reasonable in view of the half-life of this protein (48 hours). These lower percentages both in non-diabetic subjects and diabetic patients show that the protein is subject to only moderate rates of glycation. Moreover, there is enough C3 in the organism to ensure that the entire complement system is functional. Therefore, it is reasonable to assume that there was no alteration of complement function.

Such an hypothesis is supported by results for IgG glycation (5, 6). *Morin* et al. (5) found no impairment of antigen-antibody binding after in vitro glycation of non-human IgG with very high glucose concentrations. Furthermore, no variation is observed in cell agglutination, complement-mediated lymphocytotoxicity reaction between human IgG before and after in vitro glycation, even with very high glucose concentrations. Similar results were reported for eluates of glycated and non-glycated IgG.

No significant increase of immune complexes in diabetic patients was found in the present study in agreement with *Di Mario* et al. (8, 9). There is no evidence that the temporary increase of circulating immune complexes near the time of diabetes diagnosis described by some authors (7, 17) is related to glycation. This increase of the immune complexes is probably due to other impairments of immune mechanisms.

References

1. Zoppi, F., Mosca, A., Granata, S. & Montalbetti, N. (1987) Glycated proteins in serum: Effect of their relative proportions on their alkaline reducing activity in the fructosamine test. Clin. Chem. *33*, 1895–1897.
2. Brownlee, M., Cerami, A. & Vlassara, H. (1988) Advanced glycosylation and product in tissue and the biochemical basis on diabetic complications. N. Eng. J. Med. *318*, 1315–1321.
3. Dolhofer, R. & Wieland, O. H. (1980) Increased glycosylation of serum albumin in diabetes melitus. Diabetes *29*, 417–422.
4. Kaneshige, H. (1987) Non enzymatic glycosylation of serum IgG and its effects of antibody reactivity in patients with diabetes mellitus. Diabetes *33*, 822–828.
5. Morin, L. G., Austin, G. E. & Burkalter, A. (1987) Nonenzymatic glycation of immunoglobulins does not impair antigen-antibody binding. Clin. Chem. *33*, 692–694.
6. Morin, L. G., Austin, G. E., Rodey, G. E. & Johnson, J. E. (1989) Nonenzymatic glycation of human immunoglobulins does not impair their immunoreactivity. Clin. Chem. *35*, 1039–1042.
7. Abrass, C. K., Heber, D. & Lieberman, J. (1983) Circulating immune complexes in patients with diabetes mellitus. Clin. Exp. Immunol. *52*, 164–172.
8. Di Mario, U., Iavicoli, M. & Andreani, D. (1980) Circulating immune complexes in diabetes. Diabetologia *19*, 89–92.
9. Di Mario, U., Ventriglia, Iavicoli, M., Guy, K. & Andreani, D. (1983) The correlation between insulin antibodies and circulating immune complexes in diabetics with and without microangiopathy. Clin. Exp. Immunol. *52*, 575–580.
10. Austin, G. E., Mullins, R. H. & Morin, L. G. (1987) Nonenzymatic glycation of individual proteins in normoglycemic and hyperglycemic patients. Clin. Chem. *33*, 2220–2224.
11. Gaillard, O., Meillet, D., Diemert, M. C., Musset, L., Delattre, J., Schuller, E. & Galli, J. (1993) Time-resolved immunofluorometric assay of complement C3: Application to cerebrospinal fluid. Clin. Chem. *39*, 309–312.
12. Schleicher, E. & Wieland, O. H. (1989) Protein glycation: Measurement and clinical relevance. J. Clin. Chem. Clin. Biochem. *27*, 577–587.
13. Sobel, D. O. & Shakir, M. (1987) Determination of glycated plasma proteins in normal and diabetic subjects utilizing aminophenylboronic acid columns. Diab. Metab. *13*, 575–581.
14. Rendell, M., Kao, G., Mecherikunnel, P., Petersen, B., Duhaney, R., Nierenberg, J., Rasbold, K., Klenk, D. & Smith, P. K. (1985) Aminophenylboronic acid affinity chromatography and thiobarbituric acid colorimetry compared for measuring glycated albumin. Clin. Chem. *31*, 229–234.
15. Dolhofer-Bliesener, R. & Gerbitz, K. D. (1990) Impairment by glycation of immunoglobulin G Fc fragment function. Scand. J. Clin. Lab. Invest. *50*, 739–746.
16. Nerup, J. & Lernmark, A. (1981) Autoimmunity in insulin-dependent diabetes mellitus. Am. J. Med. *70*, 135–141.

Dr Olivier Gaillard
Laboratoire d'Immunochimie
Hôpital de la Salpêtrière
47, Bd. de l'Hôpital
F-75651 Paris Cédex 13
France